that KCN rapidly stopped the enzymatic production of quinone. In the absence of active enzyme, the unstable quinone then slowly disappeared from the system. In the case of curve 2, the addition of the same amount of KCN, after the O.D. *vs.* time curve had become horizontal, was also followed by a decrease in absorbance at 390 mμ. Since it is apparent from curve 3 that this amount of KCN had no effect on the fact of *o*-benzoquinone produced non-enzymatically, it is clear that the break in curve 2 was due only to the elimination of the enzymatic quinone-producing reaction. In other words, there must have been active enzyme producing quinone, and thereby maintaining a steady state, during the period that the O.D. *vs.* time curve (curve 2) was horizontal.

In the light of the foregoing results, the reactivation of color formation following the addition of ascorbic acid can be explained as a temporary smothering of the quinone-removing reaction (by lowering the quinone concentration to the point where its reaction with solvent is negligibly slow) so that the unaffected enzymatic quinone producing reaction becomes observable as an over-all rise in O.D.

When ascorbic acid is added initially to the enzyme–catechol reaction-mixture, it delays the appearance of the quinone color until it has all been oxidized. This reaction forms the basis of the chronometric method.[7] If, now, ascorbic acid here functions only as a reducing agent having no activating effect on the enzyme, the rate of browning in the solution, when the color does finally appear, should be equal (after correction for quinone loss) to the rate of color formation in a solution which is at the same stage of reaction but has had no ascorbic acid added to it.

In the experiments represented graphically in Fig. 6, varying amounts of ascorbic acid were added, at zero time, to several identical enzyme–catechol reaction systems and the eventual rate of color appearance recorded. If one compares the initial slopes of curves II, III, IV with the slope, at the same abscissa, of curve I (the blank), then it becomes apparent that Ponting's contention that ascorbic acid has an activating effect upon the enzyme, is not well founded. These results, obtained as they were during the first few minutes of reaction, constitute a good check on the validity of the chronometric method, and agree with the findings of Ingraham[15] in a recent investigation on the same subject.

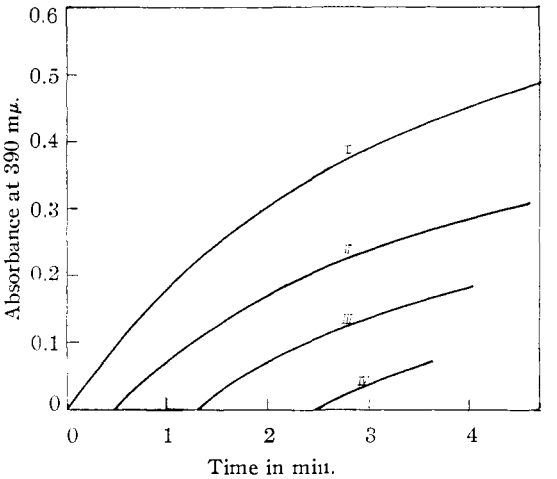

Fig. 6.—Showing the development of *o*-quinone color in tyrosinase-catechol solutions containing initially varying amounts of ascorbic acid; temp. 24.3 ± 0.5°.

Curve	1	2	3	4
Ascorbic acid, mg.	0	2.0	4.0	6.0

In each experiment a 10-ml. aliquot of a chronometric reaction mixture[6] (containing 10 ml. of 0.4 *M* phosphate–0.2 *M* nitrate buffer, *p*H 5.1, 1 ml. of 20 mg./ml. catechol solution, 0–3 ml. of 2 mg./ml. ascorbic acid solution, and copper-free water to a volume of 100 ml.) was pipetted into the special 1 cm. bubbler cell (Fig. 1) at zero time, 0.1 ml. of enzyme (3.3 units) was added and the air supply turned on. Note that the initial slopes of curves 2, 3 and 4 are equal to the slope of curve 1 at the same abscissa.

Acknowledgments.—The authors wish to express their thanks to Mr. Stanley Lewis for his painstaking efforts in preparing the enzyme. The financial assistance given by the Eli Lilly Company in support of this research is also greatly appreciated.

NEW YORK, N. Y.

(15) L. L. Ingraham, THIS JOURNAL, **78**, 5095 (1956).

[CONTRIBUTION FROM THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION]

High Molecular Weight Poly-α,L-glutamic Acid: Preparation and Optical Rotation Changes[1]

BY M. IDELSON[2] AND E. R. BLOUT[2]

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The preparation of high molecular weight (degree of polymerization >500) poly-α,L-glutamic acid is described. The changes in optical rotation upon ionization of poly-α,L-glutamic acid in water were measured and found to be qualitatively similar to those which have been observed in the denaturation of some proteins.

High molecular weight water-soluble ionic polypeptides were desired in order to examine their physical-chemical and biological properties and to compare them with those of proteins. For these purposes a high molecular weight polypeptide is defined as one having a molecular weight around 50,000 or a degree of polymerization (*DP*) of at

(1) Polypeptides XXI. For the previous paper in this series see E. R. Blout and R. H. Karlson, THIS JOURNAL, **80**, 1259 (1958).

(2) Chemical Research Laboratory, Polaroid Corporation, Cambridge 39, Mass.

least 500. In this paper we describe the preparation of high molecular weight poly-α,L-glutamic acid (L-PGA) from poly-γ-benzyl-L-glutamate (L-PBG)[3] and the optical rotatory behavior in aqueous solution. Earlier communications have outlined the preparation of L-PGA[4] and some of its properties have been reported.[5]

Synthesis.—Previous investigators have used alkaline aqueous alcohol[6,7] and sodium in liquid ammonia[6] for the removal of ester groups from polyamino acid esters; both methods lead to some racemization of the amino acids and are thus unsuited for the preparation of optically pure polymers. Catalytic hydrogenation of L-PBG to remove benzyl groups was unsuccessful.[6] Phosphonium iodide in glacial acetic acid was used in the preparation of polyaspartic acid,[8] poly-L-lysine hydroiodide[9] and L-PGA,[6,10] but phosphonium iodide is not a satisfactory reagent for larger scale preparations since it is dangerous to prepare and handle in large quantities. Using dry hydrogen bromide in glacial acetic acid, poly-L-lysine of unspecified molecular weight has been prepared from poly-ε-carbobenzyloxylysine,[11a] as well as copolymers of glutamic acid[11b] and aspartic acid[11c] from their benzyl esters; the use of this and other reagent–solvent systems for debenzylating L-PBG is reported here.

Our investigations have shown that hydrogen bromide is the most satisfactory reagent for the debenzylation of polyamino acid esters, and that the best solvents for this reaction are those which dissolve L-PBG and which do not react with hydrogen bromide. These solvents include benzene, liquid sulfur dioxide, and trifluoroacetic acid (TFA). The debenzylation of high molecular weight L-PBG proceeds to completion within a few hours at room temperature using any of the aforementioned solvents saturated with hydrogen bromide. The products (L-PGA) were shown to be free of benzyl ester groups by measurement of their ultraviolet absorption spectrum in the region in which benzyl groups absorb (230 to 270 mμ). Nitromethane also was used as a solvent for carrying out the debenzylation reaction, but hydrogen bromide reacts with nitromethane to produce bromine and ammonium bromide as two of the products. Because the solubility of L-PBG in nitromethane decreases as the molecular weight of the polymer increases, this solvent is not practical for L-PBG polymers whose MW_w exceeds about 250,000.

It was originally thought that in the presence of HBr, traces of water would cause cleavage of peptide bonds. Accordingly, considerable effort was expended to obtain completely anhydrous conditions. However, subsequent experiments showed that small amounts of water (~0.02% in the solvent) did not decrease the molecular weight of the final products.

Hydrogen bromide in glacial acetic acid has not been found to be a useful reagent:solvent pair for the preparation of L-PGA because (a) high molecular weight L-PBG is not soluble in this system and (b) lower molecular weight L-PBG's show extensive peptide bond cleavage.

Molecular weights were determined from viscosity measurements in aqueous solution at *p*H 7.3.[12] L-PGA exists, at this *p*H, in a non-helical configuration.[4,5] Molecular weight determinations on the L-PBG starting materials[3] and on the L-PGA products indicated that under our optimum conditions approximately one peptide bond is broken per thousand benzyl ester groups cleaved. Thus using L-PBG having a weight average degree of polymerization (DP_w) of 3000 the DP_w of the L-PGA should be *ca.* 750. Some results of typical debenzylation experiments are shown in Table I.

TABLE I

Prepn. no.	DP_w of L-PBG	Solvent	$[\eta]^{2M\ NaCl}_{pH\ 7.3}$ [c]	DP_w of L-PGA
36	140	AcOH	0.07[b]	50
65	1000	CH_3NO_2	0.76	540
121	3100	CH_3NO_2[a]	1.10[b]	750
462	1700	TFA	0.61	420
470	1700	SO_2	.36	250
326	250	Benzene	.33[b]	220
433	600	Benzene	.54	370
434	1100	Benzene	.84	600
435	2000	Benzene	1.04	700
419	2700	Benzene	1.17	800

[a] Plus 10% TFA. [b] By interpolation from 0.2 *M* NaCl viscosity data. [c] Based on free acid.

From the results of the experiments reported in this paper a convenient method has been developed for the preparation of high molecular weight L-PGA in kilogram quantities. In this method γ-benzyl-L-glutamate-N-carboxyanhydride is polymerized in benzene to a polymer of the desired MW_w,[3] and then the polyester is converted in the polymerization solution to the polyacid with hydrogen bromide.

Optical Rotation.—Previously it has been noted[4,5] that there is a large decrease in the levo rotation when aqueous solutions of the sodium salt of L-PGA are lowered in *p*H. Figure 1 shows a plot of $[\alpha]^{22}_{546}$ as a function of *p*H for L-PGA in (A) aqueous solution and (B) in 0.2 *M* NaCl solution. In both cases the curves have the same general shape and the specific rotation changes from approximately −120 to −8° upon changing the *p*H from 7.0 to 4.5. However in the presence of 0.2 *M* salt the entire curve is displaced about 0.5 *p*H unit toward lower *p*H's, and the $[\alpha]_{546}$ values at *p*H > 6 are about 10° smaller.

Although it does not yet appear possible to interpret the optical rotation data directly in terms of

(3) E. R. Blout and R. H. Karlson, THIS JOURNAL, **78**, 941 (1956).
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(5) P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Polymer Sci.*, **23**, 851 (1957).
(6) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).
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(11) (a) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1954); (b) M. Sela, E. Katchalski and M. Gehatia, THIS JOURNAL, **78**, 746 (1956); (c) M. Sela and E. Katchalski, *ibid.*, **77**, 3662 (1955).

(12) We are indebted to Drs. Paul Doty and A. Wada for the calibration of the molecular weight–viscosity scale for L-PGA. Absolute molecular weights of poly-L-glutamic acid were determined from light scattering measurements (A. Wada and P. Doty, to be published).

configurational changes, some conclusions may be drawn if these data are related to infrared results. In particular it has been shown[4] by means of infrared dichroism measurements that the non-ionized form of L-PGA in the solid state exhibits dichroism consistent with those of helical configurations of polypeptides. Recently we have observed similar dichroism in streaming solutions of L-PGA[13] in dioxane–D_2O which leads us to conclude that the helical configuration is preserved in solution if the L-PGA is not ionized. Similar conclusions may be drawn from the anomalous rotatory dispersion shown by such solutions.[5] We may thus assume that the small negative rotation, $[\alpha]_{546}$, shown by L-PGA is characteristic of the helical configuration and that the change in rotation (~110°) upon raising the *p*H is a result of the loss of the helical configuration caused by electrostatic repulsions due to the ionization of the γ-carboxyl groups. This change in specific optical rotation is somewhat larger than that observed in the loss of specific structure (or denaturation) of some proteins. However a reasonable explanation that has been suggested[14,15] for this smaller change in proteins as compared to L-PGA (~80° as compared to 110°) is that it is due to the lesser helical contents of proteins.

It should be noted that the changes in optical rotation of L-PGA, like the changes in infrared dichroism,[4] are completely reversible.

Finally, some comment must be made about the small but apparent increase in negative rotation of L-PGA upon decreasing the *p*H below 4.5 (Fig. 1). This change may be associated with the further de-ionization of carboxyl groups and not with the peptide backbone. The further de-ionization of the carboxyl groups may lead to a contraction of the helix by decreasing the repulsive forces on the side chains, which in turn would affect the optical rotation. One way to explore this possibility is with other water-soluble polypeptides having lower contents of ionizable groups, and these investigations are now under way.

Experimental

Materials.—Poly-γ-benzyl-L-glutamate (L-PBG) was prepared as described earlier.[3]

Solvents and Reagents.—Analytical reagent grade benzene was refluxed with calcium hydride for several hours and distilled from it immediately before use. Nitromethane was fractionally distilled through a ten-plate bubble plate column; the fraction boiling at 101–102° was used. Matheson "anhydrous" sulfur dioxide was dried further by passing through a phosphorus pentoxide drying tower and was condensed directly into the reaction vessel. Analytical reagent grade acetic acid was used without further purification (du Pont, 99.7%). Trifluoroacetic acid (Minnesota Mining and Manufacturing Co.) was fractionally distilled; the portion boiling at 71–72° was used.

Matheson anhydrous hydrogen bromide was used at first. It was dried in a phosphorus pentoxide drying tower followed by a Dry Ice trap. Due to corrosion of the tank and valves, the synthesis from hydrogen and bromine described in reference[16] was used in later experiments. The drying agents, which were included in the apparatus, were anhydrous calcium bromide followed by anhydrous aluminum bromide.

Matheson anhydrous hydrogen chloride was dried over aluminum chloride before use.

Debenzylation Reactions.—Because each solvent for the debenzylation reaction required somewhat different conditions, a typical reaction in each solvent is described in detail.

A. **Nitromethane.**—One gram of L-PBG (MW_w 220,000) was stirred vigorously for 10 minutes in 50 ml. of nitromethane to dissolve as much as possible and to break up the remainder into a fine suspension. Hydrogen bromide bubbled into the mixture caused the undissolved polymer to dissolve after about 5 minutes. Several minutes later the product began to precipitate, and after 0.5 hour the hydrogen bromide stream was stopped. After another hour a sample of the precipitate of poly-α,L-glutamic acid (L-PGA) was soluble in sodium bicarbonate solution. The L-PGA was collected and extracted with ether in a Soxhlet extractor for several hours, and finally dried. The yield was 0.62 g. (100%), 0% benzyl (by ultraviolet absorption of solution at 258 mμ), $[\eta]^{2\ M\ Na_2SO_4}_{2H\ 7.3}$ 0.76.

B. **Acetic Acid.**—Glacial acetic acid is a poor solvent for L-PBG. One gram of L-PBG, MW_w 30,000, was stirred with 100 ml. of glacial acetic acid at room temperature for 45 minutes, but it did not dissolve. However, L-PBG of this MW_w was found to be soluble in hot acetic acid. Therefore the hydrogen bromide was bubbled through the hot solution for 0.5 hour. After standing 5 hours, excess hydrogen bromide was removed on a water-pump. The acetic acid was then frozen and removed by freeze-drying. The solid product was extracted with acetone and dried, $[\eta]^{2\ M\ NaCl}_{2H\ 7.3}$ 0.07. It contained 25% of benzyl groups.

L-PBG, MW_w 375,000, was not soluble even in boiling acetic acid. After bubbling hydrogen bromide through a suspension of 1 g. of L-PBG in 100 ml. of acetic acid for 0.75 hr., and standing for 4 hr., the supernatant liquid was decanted from the undissolved polymer. The supernatant yielded after freeze-drying 0.1 g. of acetone extracted material, $[\eta]^{0.2\ M\ NaCl}_{2H\ 7.3}$ 0.22. It contained 23% of benzyl groups. The acetic acid-insoluble fraction was not soluble in dilute sodium bicarbonate and, therefore, it may be concluded that it was not debenzylated.

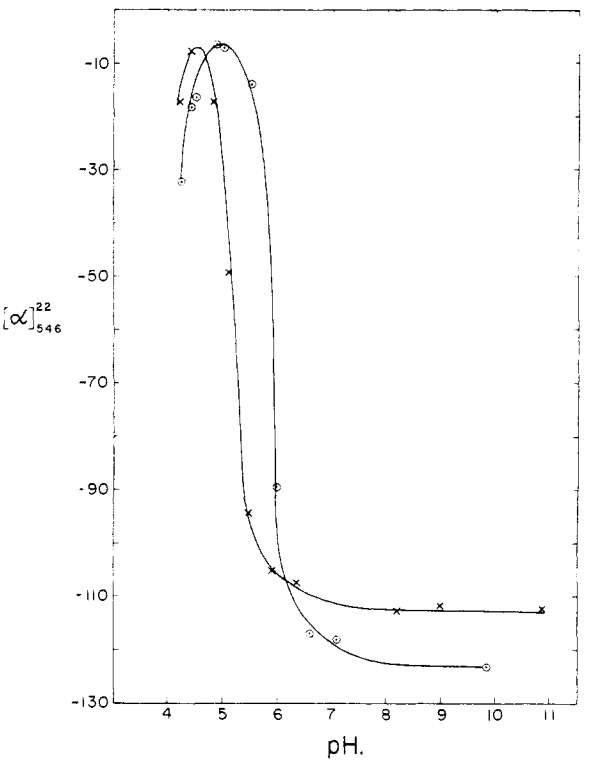

Fig. 1.—The optical rotation, $[\alpha]^{22}_{546}$, of poly-α,L-glutamic acid (#186C, 80% Na salt) as a function of *p*H: -⊙-⊙-⊙-, in water; -X-X-X-, in 0.2 *M* NaCl.

(13) G. R. Bird and E. R. Blout, unpublished results.

(14) C. Cohen, *Nature*, **175**, 129 (1955), and previous references reported therein.

(15) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).

(16) J. R. Ruhoff, R. E. Burnett and E. E. Reid, "Organic Syntheses," Coll. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 338.

C. **Trifluoroacetic Acid.**—One-half gram of L-PBG (MW_w 680,000) was dissolved in 25 ml. of trifluoroacetic acid. Hydrogen bromide was bubbled into the solution for 15 minutes, and the reaction was allowed to stand for 2 hours. It was then poured into several volumes of ether, filtered and extracted with ether in a Soxhlet extractor, giving 0.25 g. of product, $[\eta]^{0.2\ M\ Na_2SO_4}_{pH\ 7.3}$ 1.4.

In later preparations, *e.g.*, those reported in the table, the trifluoroacetic acid was removed by freeze-drying (KOH trap to protect pump) after eliminating most of the hydrogen bromide on the aspirator. The L-PGA then was extracted with ether or acetone to remove benzyl bromide.

D. **Benzene.**—This is the most convenient solvent for preparing L-PGA. γ-Benzyl-L-glutamate NCA was polymerized in benzene at 1% concentration as described.[3] For MW_w determination a sample of the viscous polymer solution was then removed and the L-PBG isolated by precipitation into ethanol. A gas inlet tube bearing a calcium chloride drying tube on the vent was attached to the flask containing the remainder of the polymer solution. Hydrogen bromide was then bubbled into the solution for about an hour, after which the solution became a thick gel. It was allowed to stand overnight, and the supernatant benzene was drawn from the reaction flask by means of an aspirator the next day. The L-PGA usually is a small, hard lump at this time. If it is not, the reaction should be allowed to stand until the L-PGA has become a hard lump; if the reaction is stopped before this time, the product will be incompletely debenzylated. After removing the bulk of the benzene, the flask was placed first on aspirator vacuum to eliminate most of the hydrogen bromide, and then on a vacuum pump (use KOH trap) to evaporate the remaining benzene. The L-PGA was extracted with acetone in a continuous extractor until all the colored by-products and benzyl bromide were removed, and finally dried. The yield was the theoretical, and the results of several experiments are given in Table 1.

E. **Sulfur Dioxide.**—A 125-ml. pressure bottle containing 2 g. of L-PBG (MW_w 680,000) was dried at 110°. Anhydrous sulfur dioxide (50 ml.) was condensed into it in a Dry Ice-bath (protected by a calcium chloride drying tube); the bottle was closed and allowed to warm to room temperature until the polymer dissolved. It was then cooled again in Dry Ice, opened, and hydrogen bromide bubbled in for 15 minutes (drying tube). The bottle was closed again and allowed to stand two hours at room temperature. After cooling again in Dry Ice, it was opened and the sulfur dioxide poured out, leaving the L-PGA behind. The rest of the SO_2 evaporated and the product was washed thoroughly with ether, leaving 1.2 g. of L-PGA containing no benzyl groups and having $[\eta]^{2\ M\ Na_2SO_4}_{pH\ 7.3}$ 1.64.

Anal. Calcd. for $C_5H_7NO_3$: C, 46.5; H, 5.4; N, 10.8. Found: C, 46.8; H, 5.9; N, 10.2.

Optical Rotation Measurements.—Optical rotations were measured with a Rudolph high precision photoelectric polarimeter, model 200, using a General Electric H-100-A4 mercury lamp as the light source. The 546 line was isolated with a Baird interference filter in conjunction with Corning Glass filters #9780 and #3484. Measurements were made at 22°; concentrations were 0.2%.

The sample of L-PGA used for the optical rotation measurements was #186-C prepared by method D. It was dialyzed for 24 hours at *p*H 7.3 then lyophilized, $[\eta]^{0.2\ M\ NaCl}_{pH\ 7.3}$ 0.93, DP_w 630, MW_w 82,000.

Anal. Calcd. for 80% sodium salt and 20% free L-PGA: C, 40.9; H, 4.3; Na, 12.2. Found: C, 40.4; H, 4.3; Na, 12.0.

Acknowledgment.—We are pleased to acknowledge the support of this work by the Office of the Surgeon General, Department of the Army, and the valuable technical assistance of Misses Evelyn DesRoches and Ann Gounaris in the preparative work, and Mr. Kenneth Norland in the optical rotatory measurements.

BOSTON 15, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Kinetic and Equilibrium Measurements of the Regeneration of Acid-denatured Horse Ferrihemoglobin[1]

BY JACINTO STEINHARDT, ETHEL M. ZAISER AND SHERMAN BEYCHOK[2]

RECEIVED APRIL 3, 1958

Measurements are presented of the rates of denaturation and regeneration of horse ferrihemoglobin over more than one *p*H unit in formate buffers at 0.2°. The kinetics of denaturation (which is accompanied by a large increase in acid-binding groups) are first-order and uncomplicated, as previously reported for higher temperatures. Evidence is presented that the product formed varies continuously as a function of the *p*H and temperature of denaturation. Rate measurements on regeneration, now made in a direct manner for the first time, show that regeneration, although first-order also, yields first an intermediate product, spectroscopically similar to native protein but characterized by its effect of inhibiting the reaction by which it is formed. The inhibitor reverts, on standing, to apparently normal native protein which can be denatured again at the original rate. The highly anomalous kinetics of regeneration are uniquely explained by the existence of this inhibitor, which is also demonstrated directly by experiments devised for that purpose. The rate of the regeneration reaction, contrary to earlier reports, is independent of *p*H. Equilibrium data are given, including values of ΔH, as are temperature coefficients for two of the underlying reactions. The difficulties of interpreting the equilibrium, or of relating ΔH to the activation energies measured, are discussed in detail.

I. Introduction

Previous papers[3-5] have reported that when horse ferrihemoglobin is denatured by acid, in the *p*H range 3.0–4.2, the reaction is accompanied by an increase in its acid-binding capacity which corresponds to the addition (in one step) of about 36 basic groups per molecule. The identity of the new basic groups has been discussed at length elsewhere[6]; the evidence strongly favors liberation of an equal number of carboxylate (or carboxylate plus a few imidazole) and lysine ε-amino or guanido groups. At the *p*H of denaturation the lysine or guanido groups immediately combine with protons from carboxyl groups, so that the over-all effect is an apparent increase in carboxylate only. This increase, like the denaturation itself,[7] is almost

(1) A brief account of this work was reported at the meeting of the American Chemical Society in New York on September 12, 1957. Some of the data were presented at the meeting of the American Society of Biological Chemists at Atlantic City, N. J., in April, 1954.

(2) Certain of the measurements in this paper were made by Robert J. Gibbs, whose assistance is here gratefully acknowledged.

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(5) E. M. Zaiser and J. Steinhardt, *ibid.*, **76**, 2866 (1954).

(6) J. Steinhardt and E. M. Zaiser, *Advances in Protein Chem.*, **10**, 151 (1955).

(7) Denaturation was followed quantitatively by both changes in absorption at 406 mμ and by changes in solubility at the isoelectric point.